

HIV-1 Tat protein exits from cells via a leaderless secretory pathway and binds to extracellular matrix-associated heparan sulfate proteoglycans through its basic region

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Objective: To analyze the mechanisms of release and the extracellular fate of the HIV-1 Tat protein and to determine the Tat domain binding to the extracellular matrix.

Design and methods: Release of Tat was studied by pulse-chase experiments with Tat-transfected COS-1 cells in the presence or absence of different serum concentrations, temperatures and drugs inhibiting the classical secretion pathway or endo-exocytosis, such as brefeldin A and methylamine. The binding of extracellular Tat to heparan sulfate proteoglycans (HSPG) was determined by using trypsin, heparin or heparinase in pulse-chase experiments, by gel shift and competition assays with radiolabeled heparin, and by heparin-affinity chromatography. The mapping of the Tat binding site to heparin was defined by functional assays of rescue of Tat-defective HIV-1 proviruses.

Results: Tat is released in the absence of cell death or permeability changes. Tat release is dependent upon the temperature and serum concentration, and it is not blocked by brefeldin A or methylamine. After release, a portion of the protein remains in a soluble form whereas the other binds to extracellular matrix (ECM)-associated HSPG. The HSPG-bound Tat can be retrieved into a soluble form by heparin, heparinase or trypsin. Binding to heparin is competed out by heparin-binding factors such as basic fibroblast growth factor (bFGF), and it is mediated by the Tat basic region which forms a specific complex with heparin which blocks HIV-1 rescue by exogenous Tat and allows purification of a highly biologically active protein.

Conclusions: These results demonstrate that Tat exits from intact cells through a leaderless secretion pathway which shares several features with that of acid FGF or bFGF. The released Tat binds to HSPG through its basic region and this determines its storage into the ECM, as occurs for bFGF.

et al.
AIDS 1997, 11:1421-1431

Keywords: Tat, extracellular, release, heparin binding, proteoglycans, AIDS, Kaposi's sarcoma

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Sponsorship: Supported in part by a grant from the IX AIDS Project of the Italian Ministry of Health.

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Date of receipt: 3 April 1997; revised: 2 June 1997; accepted: 6 June 1997.

Introduction

The Tat protein of HIV-1 is an early transactivator of viral gene expression and replication [1-4]. Tat is a small polypeptide of 86-102 amino acids, depending on the viral strain, that is encoded by two exons. The first exon encodes a 72-amino acid peptide that is conserved in all viral isolates, possesses a full transactivation function [5,6] and contains an arginine-lysine-rich basic region (amino acids 49-57) which is required for nuclear localization and nucleic acid binding of Tat. The C-terminal amino acids (73-86), encoded by the second Tat exon, are not required for transactivation. However, this region contains the arginine-glycine-aspartic acid (RGD) motif that is also present in extracellular matrix (ECM) proteins such as fibronectin and vitronectin and mediates cell adhesion and binding of extracellular Tat to integrin receptors expressed by activated endothelial cells, CD4 T cells and other cell types [7-11].

In addition to the transactivation of HIV-1 gene expression, Tat can modulate the expression of many cellular genes including those for cytokines, adhesion molecules, major histocompatibility complex class I proteins and oncogenes, as well as cellular functions such as cell survival, growth and angiogenesis [5,6]. In addition, in transgenic mice, Tat can induce the development of tumors [12,13].

Recent data indicate that Tat can act as both an intracellular and an extracellular protein and can induce effects through different but co-operating pathways that, at least in part, depend upon protein concentration and target cell type. For example, extracellular or exogenous Tat can be taken up by cells and activate HIV-1 gene expression in infected cells [14-19]. Extracellular Tat also promotes the growth, migration, invasion and adhesion of Kaposi's sarcoma (KS)-derived endothelial spindle cells, cytokine-activated normal endothelial cells and endothelial tumor cell lines [9,18,20-24]. By these activities and by promoting angiogenesis, Tat can increase the frequency and the aggression of KS in infected individuals [10,25].

The HIV-1 transactivation effect of extracellular Tat is observed at nanomolar concentrations of the protein. In contrast, the effects on KS and vascular cells are observed at picomolar concentrations of Tat, and are mediated by both the basic and the RGD region of the protein. In particular, the RGD motif of Tat binds the integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$, the receptors of fibronectin and vitronectin, respectively [9-11] (G. Barillari, personal communication, 1996), suggesting that extracellular Tat can mimic the effect of ECM proteins in regulating cell survival, growth and angiogenesis.

Other data have shown that extracellular Tat can induce apoptosis of T cells from uninfected donors and

that this is associated with enhanced activation of cyclin-dependent kinases [26]. Tat can also amplify the cytotoxic effect of tumor necrosis factor by altering the cellular redox state [27] and can increase T-cell receptor and CD4 (gp120)-induced apoptosis by up-regulating CD95 ligand expression [28]. This may lead, at least partially, to the T-cell depletion present in AIDS. However, Tat can also protect cells from apoptosis under serum-free conditions [29-31], and can modulate CD4 T-cell survival and growth induced by anti-CD3 costimulation [11]. Also these effects of Tat are mediated by both the basic and the RGD region, and they may depend on the cell system, the form of Tat (expressed intracellularly or added to the cells) and protein concentration. Thus, extracellular Tat may play a role in the pathogenesis of AIDS and associated malignancies. Therefore, the study of its release and extracellular fate is a focal point in understanding the biological relevance of Tat effects and evaluating potential therapeutic strategies.

Our previous data indicated that HIV-1 acutely infected T cells or *tat*-transfected COS-1 cells release substantial quantities of biologically active Tat prior to or in the absence of cell death, respectively, and at the moment of the highest gene expression [18,23]. In addition, mutations in the basic region of Tat increase its cytoplasmic concentration and release [18]. This extracellular Tat can be taken up by neighboring cells, localize in the nuclei, transactivate HIV-1 gene expression, or activate the replication of Tat-defective HIV-1 proviruses, or can induce cell growth [18]. Tat has also been detected in sera of HIV-1-infected individuals and at levels that are equivalent to those found in supernatants of HIV-1-infected cells [28]. In addition, extracellular Tat has been detected in AIDS-KS lesions where it co-stains with endothelial spindle cells expressing the β_1 or β_3 integrins [10]. These data indicate that effective Tat concentrations can be reached *in vivo* and that Tat can bind and/or can be taken up by neighboring cells. In fact, uptake of Tat is observed with most cell types. In addition, Tat can also be a carrier for other molecules and can mediate a high efficiency uptake into cells [32-34].

Most of the pre-secretory proteins contain N-terminal leader or signal sequences of 13-30 hydrophobic amino acids that mediate translocation of the proteins across the endoplasmic reticulum (ER) to the Golgi apparatus that they leave in secretory vesicles. These fuse with the plasma membrane and are released extracellularly. However, there are many exceptions to this. Some proteins with defined extracellular functions but devoid of a signal sequence are released by an alternative secretory pathway independent of the ER-Golgi complex. These include interleukin (IL)-1 β [35], lectin-14 [36], acid fibroblast growth factor (aFGF) [37,38], basic FGF (bFGF) [39-41] and thioredoxin [42]. Similarly,

although Tat protein has no signal sequence, it is released as a biologically active protein and exerts extracellular functions.

Here we demonstrate that release of Tat occurs in the absence of cell death and is through a leaderless secretion pathway which shares several features with that utilized by IL-1 β , aFGF, or bFGF. In addition, as for bFGF, released Tat binds heparan sulfate proteoglycans (HSPG) via its basic region that has heparin-binding properties. This determines the storage of Tat into the ECM. This Tat can be retrieved in a biologically active form by heparin.

Materials and methods

Plasmid DNA and transfection of COS-1 cells

The plasmid pCVTAT containing the full-length *tat* gene and the control vector pCV0 have been described previously [18]. The COS-1 cells were cultured in RPMI-1640 (Gibco BRL, Gaithersburg, Maryland, USA) containing 10% fetal calf serum (FCS) (10% FCS-RPMI) until 90% confluence. Cells were then gently trypsinized, washed with phosphate-buffered saline (PBS) without Ca²⁺ or Mg²⁺ and resuspended in 1.6 ml of electroporation buffer [272 mM sucrose, 7 mM potassium phosphate (pH 7.4), 1 mM magnesium chloride]. The cell suspension (800 μ l) was gently mixed with the plasmid DNA (30 μ g) and incubated on ice for 8–10 min. After electroschock (25 μ F, 0.28 kV), cells were incubated again on ice for a further 8–10 min and gently transferred to a 75 cm² flask. By this method, cell viability is > 99% and no cellular hyper-permeability is detected [18 and see below].

Determination of cell viability

Cell viability was evaluated by trypan blue dye exclusion. Cell membrane permeability changes by the determination of lactic dehydrogenase (LDH) activity in culture supernatants of transfected cells (48 h after transfection, during labeling or at the end of the period of chase). Growth media and supernatants from 1, 5 and 10% of lysed cells were used as the negative and positive controls (for the standard LDH curve), respectively. Determination of LDH levels represents the most sensitive method to detect cell damage and non-specific exit of cellular proteins [18,35].

Metabolic labeling

At the moment of the highest gene expression (36–48 h post-transfection), transfected cells were incubated with methionine- and cysteine-free RPMI-1640 containing 1 mM glutamine and 5 or 10% dialyzed FCS for 1 h and were labeled with 200 μ Ci/ml of [³⁵S]methionine and [³⁵S]cysteine in the same medium for 5 h at 37°C. Supernatants were then harvested. For

pulse-chase experiments, cells were incubated with methionine- and cysteine-free medium containing 1 mM glutamine and 5 or 10% dialyzed FCS for 1 h and then pulsed for 30–90 min at 37°C with 400 μ Ci/ml of [³⁵S]methionine and 800 μ Ci/ml of [³⁵S]cysteine. After removal of the labeling medium, cells were washed three times with RPMI containing 5 or 10% of FCS and chased for different periods of time at 18, 37, or 42°C for temperature dependency experiments, or chased in the presence or absence of 0.5 μ g/ml brefeldin A or 10 mM methylamine. Cells were pre-incubated with methylamine for 1 h before the pulse. To detect bound Tat, labeled cells were washed once or twice with PBS and treated with 0.5 ml trypsin (0.05%) for 2 min, with 1 ml heparin (5 μ g/ml), or 1 ml heparinase (0.5 U/ml) for 20 min at room temperature as described previously [40]. Supernatants were then harvested after addition of 4 or 4.5 ml of 10% FCS-RPMI. Supernatants from both treated and untreated cells were then centrifuged at low speed (200 g) for 10 min to remove possibly detached cells without breaking or damaging them, transferred to a new tube and centrifuged at low speed (400 g) for another 30 min at 4°C to clear the supernatants. Labeled cells were washed in cold PBS and lysed in extraction buffer containing 0.1 M Tris (pH 8.0) 0.1 M NaCl, 0.5% nonidet-P-40 (NP-40), 0.5% deoxycholic acid and 1% aprotinin. Cell debris was removed by centrifugation at 10 000 g for 30 min at 4°C. Supernatants and cell lysates were subjected to radio-immunoprecipitation analysis (RIPA).

RIPA and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Aliquots from cell lysates and supernatants, adjusted by intracellular trichloroacetic acid (TCA) precipitable radioactivity, were precleared by preincubation for 4 h at 4°C with normal rabbit serum, followed by RIPA with the polyclonal affinity-purified anti-Tat antibody previously described [18]. The immunoprecipitates were washed four times in 'NETA' buffer (0.15 M NaCl, 0.05% NP-40, 50 mM Tris-HCl, pH 7.4, 50 mM ethylene diamine tri-acetic acid, 1% aprotinin), twice in NETA buffer with high salt (0.5 M NaCl), denatured in Laemmli buffer and separated by a 16% SDS-PAGE, that was treated with Enlightening (DuPont NEN, Boston, Massachusetts, USA), dried and exposed to Kodak X-Omat or Bio-Max film (Kodak, Rochester, New York, USA) at -70°C.

HIV-1 rescue assays

The HLM-1 cell line containing an integrated non-reversible Tat-defective provirus was used for rescue assays [18,43]. In this provirus (HXB2), the initiation codon of *tat* has been mutated to a termination codon. HLM-1 cells were seeded in 12-well culture plates at 2×10^5 cells/well, cultured for 24 h and Tat protein (2 μ g/ml) and/or heparin and/or Tat peptides (amino

acids 11–24, 46–60, or 65–80) were added to the cells. The supernatants were collected 72 h later for p24 antigen capture assays as described previously [18]. The biological activity of recombinant Tat protein collected from heparin-affinity chromatography was also tested by this assay.

Gel shift assay

Various amounts of biologically active Tat (10, 100, or 1000 ng) were mixed with 0.01 μ Ci of [*N*-sulfonate-³⁵S]heparin sodium (0.44 mCi/g, Amersham, Arlington Heights, Illinois, USA) in RPMI at 28°C for 5 min and then treated with or without 2.5 μ l heparinase (0.1 U/ μ l) at 28°C for 10 min. Competition experiments were performed by mixing bFGF (100 ng) or bovine serum albumin (BSA) (1 or 10 μ g) with 0.01 μ Ci [*N*-sulfonate-³⁵S]heparin sodium in RPMI in the presence or absence of Tat (75 ng) and then treating with 2.5 μ l heparinase (0.1 U/ μ l). Glycerol (2%) was added and then the mixture was loaded into 8% non-denaturing polyacrylamide gel and signal recorded on an autoradiogram.

Tat protein and anti-Tat antibody

The Tat protein used in the rescue and gel shift experiments was expressed in *Escherichia coli* and isolated by successive rounds of high-pressure liquid chromatography and ion-exchange chromatography as described previously [18,44]. The purified Tat protein is > 95% pure as tested by SDS-PAGE and has full biological activity as tested by rescue assays. The Tat protein was stored lyophilized at -70°C and resuspended in degassed buffer [PBS containing 0.1% BSA and 0.1 mM dithiothreitol (DTT)] before use. The plasticware was previously rinsed in PBS-BSA buffer or 10% FCS-RPMI for each procedure involving the use of Tat.

Rabbit polyclonal anti-Tat antibodies were raised against recombinant purified Tat protein and affinity purified as described previously [18,23].

Purification of recombinant Tat protein by heparin affinity chromatography

Escherichia coli (RB179 *syn-tat*) cells (10 g wet weight) expressing Tat were sonicated in 40 ml of lysis buffer [20 mM sodium phosphate, pH 7.8, 2.5% glycerol, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM DTT, 50 mM mannitol, 10 mM ascorbic acid and 500 mM NaCl] using an Ultrasonic Liquid Processor (Model XL2020, Heat Systems Inc., Cambridge, Massachusetts, USA) with three 20-sec bursts. The lysate was then clarified by centrifugation at 12 000 g for 30 min and the supernatant was incubated for 1 h at room temperature with 2 ml of heparin-Sepharose pre-washed with lysis buffer. The heparin-Sepharose was then packed into a glass column and washed with lysis buffer until no protein was detected in the wash. The

bound material was eluted with lysis buffer containing 2 M NaCl and the eluate was collected in 1 ml fractions. The homogeneity of the eluted protein was analyzed by SDS-PAGE and the biological activity was tested by the rescue assay.

Results

Release of Tat

To analyze the mechanism of Tat release, COS-1 cells were transfected with the *tat* expressing plasmid pCVTAT or the control vector pCV0 by an electroporation method described previously [18,23], which has been optimized to obtain a high efficiency of transfection in the absence of cell death or permeability changes [18]. Tat was detected both in cell extracts and in supernatants at the moment of the highest gene expression (36–48 h post-transfection) and in the absence of cell death as measured by trypan blue exclusion (Fig. 1a). To exclude the possibility that an increase of cell membrane permeability was responsible for the release of Tat, the levels of LDH activity were measured in the supernatants from transfected cells. This represents a very sensitive method to detect very early cell permeability changes and non-specific exit of intracellular proteins [18,35]. No increase of LDH was observed in transfected cells and its level was similar to that of growth medium (Fig. 1a). Similarly, no changes in the morphology of the *tat*-expressing cells were observed by staining with anti-Tat antibodies (data not shown) [18]. Finally, release of Tat was much lower or undetectable when cell death was present (data not shown). This occurs also for the release of bFGF [40,41] and indicates that cell viability and function are required for the release of Tat.

To gain insights into the possible mechanisms of Tat release, both the inhibitor of the classical secretion pathway brefeldin A, known to prevent the egress of secretory molecules from the ER [45], and the inhibitor of the endocytotic and exocytotic pathway methylamine [46], were employed. To avoid potential effects on protein synthesis, *tat*-transfected cells were pulsed for 40 min with [³⁵S]methionine and [³⁵S]cysteine and then chased for 3 h in the presence or absence of the drugs. Treatment with brefeldin A did not decrease the level of Tat present in the cell supernatants, whereas it reduced the protein species over 46 kDa that are not related to Tat (data not shown). This confirmed that release of Tat follows a pathway alternative to the classical secretory pathway. However, methylamine, a drug known to inhibit the release of IL-1 β , thioredoxin and bFGF, also did not inhibit Tat release (data not shown). This is similar to recent results determining that the release of aFGF in response to

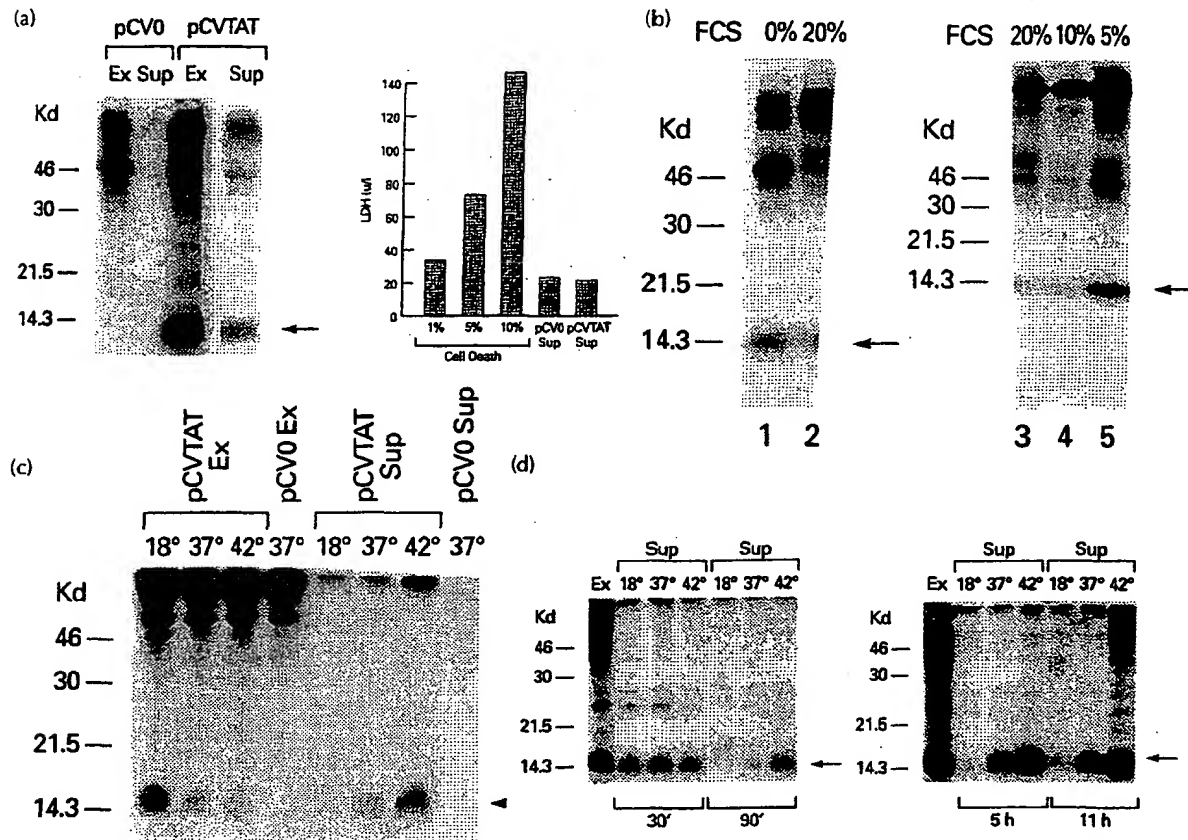


Fig. 1. (a) Tat is released from *tar*-transfected COS-1 cells in the absence of cell death. COS-1 cells were transfected with pCVTAT or the control vector pCV0. Cell extracts (Ex) or supernatants (Sup) were precipitated with polyclonal anti-Tat antibodies. Arrow indicates the Tat protein band. Left panel shows the autoradiogram. Right panel shows the level of lactic dehydrogenase (LDH) activity (U/l) measured in supernatants from transfected COS-1 cells or corresponding to 1, 5 or 10% dead cells (positive control). (b) Tat release is dependent upon serum concentrations. The transfected COS-1 cells were labeled for 5 h in the presence (20%) or absence (0%) of FCS (left panel) or in the presence of serial concentrations of FCS (5, 10 and 20%, right panel). (c) Tat was immunoprecipitated from cell extracts (Ex) or supernatants (Sup) of pCVTAT- or pCV0-transfected COS-1 cells that were labeled at 37°C for 90 min and then chased at 18, 37, or 42°C for 11 h. Cell death was <1% for all transfected cells. (d) Pulse-chase experiments with COS-1 cells transfected with pCVTAT plasmid. Tat was immunoprecipitated from cell extracts or supernatants of pCVTAT-transfected COS-1 cells pulsed for 90 min at 37°C and chased at 18, 37, or 42°C for 30 min, 90 min, 5 h, or 11 h. At each timepoint, supernatants were removed and replaced with fresh medium for another 1, 3.5, or 6 h incubation. Cell death was absent.

heat shock is not inhibited by methylamine [38]. With both drugs the LDH levels in the cell supernatants (measured at the end of the chase) were unchanged, confirming that cell death or cell damage did not account for Tat release. These results indicated that release of Tat is through a leaderless pathway, however, there are differences with release of IL-1 β and bFGF which can be inhibited by methylamine.

Scrum-free conditions and low temperature (18°C) are known to block endo- and exocytosis and to inhibit secretion of IL-1 β [35] and bFGF [39,40]. To study the serum effect, *tar*-transfected cells were pulsed in RPMI

containing 5% dialyzed FCS for 40 min and then chased in medium containing 0, 5, 10 or 20% FCS for 3 h (Fig. 1b). The release of Tat was inversely related to the serum content (0 or 5%), although cell viability or permeability (LDH levels) remained unchanged. This is the opposite of the effect of serum on the release of IL-1 β or bFGF, and suggests that the reduction of a serum component increases the release of Tat or the level of its soluble (detectable) extracellular form (see below).

A number of drugs that share the common feature of inducing synthesis of heat-shock proteins have been

demonstrated to increase the secretion of IL-1 β , aFGF, or bFGF. In addition, the release of these molecules is increased in cells exposed at 42°C and decreased at 18°C [35,37–39]. The effect of temperature on Tat release was investigated by pulse-labeling the *tat*-transfected cells at 37°C for 90 min and then by chasing them at 18, 37 and 42°C for 11 h (cumulative release of Tat) (Fig. 1c), or for 30 min, 90 min, 5 h and 11 h to analyze the kinetics of Tat release (Fig. 1d). As shown in Fig. 1c, higher temperatures increased Tat release and more Tat was released at 42°C as compared with 37°C. In contrast, low temperatures (18°C) reduced release of Tat. At the same time the intracellular content of Tat decreased or increased with high or low temperature, respectively. The kinetics experiments (Fig. 1d) indicated that Tat protein is already detectable in the supernatants of transfected cells after 30 min of chasing. At this time the level of release is not modified by different temperatures. However, at longer periods of time (≥ 90 min), the heat induced Tat release that continued to increase even after 11 h, although cell viability or LDH levels in the supernatants were unchanged. Thus, as for IL-1 β , aFGF, or bFGF, heat shock induces the release of Tat whereas low temperatures reduce it.

Soluble and HSPG-bound extracellular Tat

To investigate further the release and the fate of extracellular Tat protein, experiments were performed to determine whether, after release, Tat could associate with the cell membrane and the ECM. For this pur-

pose, the *tat*-transfected COS-1 cells were labeled for 5 h, supernatants were harvested and cells washed and treated with trypsin for 2 min at room temperature as described previously [40]. The trypsin-treated supernatants were then harvested, centrifuged at low and high speed and measured for LDH activity. Supernatants (before and after trypsin digestion) were then immunoprecipitated with anti-Tat antibodies. Tat was detected at similar levels in supernatants before and after trypsin treatment (Fig. 2a). This indicated that a fraction of released Tat binds the ECM and that this bound Tat can be retrieved by trypsin, as found for bFGF [40]. This bound Tat represented approximately one-half of the total extracellular Tat. In addition, as shown in Fig. 2a, after trypsin treatment two Tat bands were observed, the smaller extra band (about 9 kDa) representing a cleavage product at Lys-Arg residues (amino acid 51–52) [47].

It has been demonstrated that HSPG can be released from the cell surface by mild trypsinization of the cells [48]. HSPG are a common constituent of ECM, are widely distributed in tissues as basement membrane components and are also found as components of cell membrane as perimembrane or intercalated membrane of many cell types [49]. It has also been demonstrated that secreted HSPG or heparin bind extracellular bFGF and protect it from proteolytic degradation [48,50]. Finally, heparin can bind Tat and can increase or block Tat effects [8,21,47] (G. Barillari, personal communication, 1996). As heparin or heparinase can retrieve

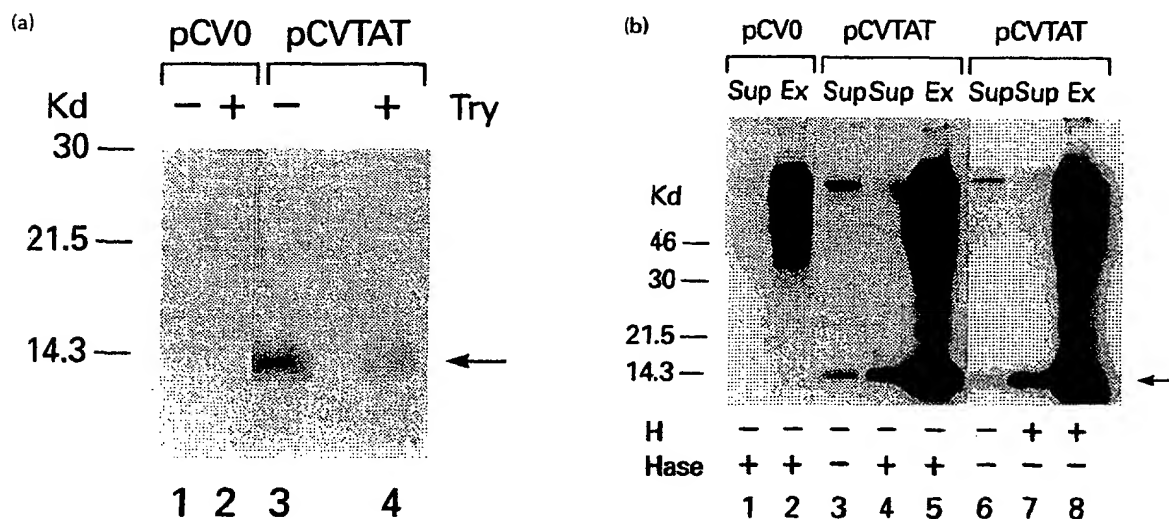


Fig. 2. Released Tat is found in a soluble and in a heparin sulfate proteoglycan (HSPG)-bound fraction. (a) The bound fraction can be retrieved by trypsin (Try). After labelling the supernatants were harvested (lanes 1 and 3) and cells washed with phosphate-buffered saline (PBS) and treated with 0.5 ml trypsin (0.05%) at 37°C for 2 min. Fresh medium (4.5 ml) was added and the supernatants were harvested again (lanes 2 and 4). (b) The bound fraction can be retrieved by heparin (H) or heparinase (Hase). After labelling, the supernatants were harvested (lanes 3 and 6) and cells washed with PBS and treated with 1 ml heparin (5 μ g/ml) or 1 ml heparinase (0.5 Unit/ml) at room temperature for 20 min. Fresh medium (4 ml) was added and supernatants were harvested (lanes 1, 4 and 7). Cell death was < 1% in all experiments.

Table 1. Heparin blocks the viral effect of extracellular Tat protein in a dose-dependent fashion.

Tat (2 µg/ml)	Heparin (µg/ml)	p24 (pg/ml)	Inhibition (%)
Preincubation with heparin			
+	-	572 ± 19	0
+	0.05	550 ± 66	4
+	0.1	588 ± 16	0
+	0.5	102 ± 1	82
+	1	10 ± 6	98
+	2.5	4 ± 1	99
+	5	17 ± 19	97
-	5	0	
No preincubation			
+	-	630	0
+	0.1	616	2
+	0.5	269	57
+	1	12	98
+	2.5	5	99
+	5	8	99
-	5	0	

Results are the average of duplicate wells from two experiments ± SD. The % inhibition of viral rescue (p24 antigen production) are shown.

HSPG-bound bFGF into a soluble form [40], similar experiments were performed with *tat*-transfected cells.

Tat-transfected cells were treated with heparin or heparinase at room temperature for 20 min. Bound Tat was released both by heparin and by heparinase (Fig. 2b). In addition, the amount of bound Tat retrieved into a soluble form by heparin or heparinase was higher than the soluble fraction suggesting that larger amounts of extracellular Tat are bound to HSPG. Although more Tat is retrieved by heparin or heparinase than by trypsin (Fig. 2a, b) this may be due to the longer time of chasing with heparin or heparinase (20 min versus 2 min with trypsin). These results indicated that extracellular Tat is present in a soluble fraction and in a fraction bound to the HSPG of the cell surface and ECM.

Heparin blocks Tat functioning by binding to the Tat basic region

The previous results indicated that Tat protein binds to HSPG and that exogenous heparin increases soluble Tat by retrieving it from these sites via a competitive effect. To analyze further the binding of heparin and Tat and the effect of this interaction on the viral effect of extracellular Tat, HIV-1 rescue assays were performed in the presence or absence of heparin. For this purpose the HLM-1 cell line was utilized that contains a Tat-defective HIV-1 provirus whose replication is induced by exogenous Tat [18]. Exogenous Tat (2 µg/ml) was mixed with various concentrations of heparin in medium at 4°C for 2 h and then added to HLM-1 cells. Viral rescue was then determined by measuring p24 content in the cell supernatants collected after 72 h by an antigen capture assay. The viral rescue induced by exogenous Tat was inhibited by

Table 2. Competition of the viral blocking effect of heparin by Tat peptides.

Tat (2 µg/ml)	Heparin (µg/ml)	Tat peptide (molar excess)*	p24 (pg/ml)	Inhibition of viral rescue (%)
+	-	-	405 ± 63	0
+	+	-	0	100
+	+	46-60 (320x)	425 ± 62	1
+	+	46-60 (160x)	350 ± 89	14
+	+	46-60 (80x)	249 ± 87	39
+	+	46-60 (40x)	33 ± 33	92
+	+	65-80 (320x)	0	100
+	+	65-80 (160x)	0	100
+	+	65-80 (80x)	10 ± 16	97.5
+	+	65-80 (40x)	21-26	94.8
+	+	11-24 (320x)	0	100
+	+	11-24 (160x)	0	100
+	+	11-24 (80x)	0	100
+	+	11-24 (40x)	0	100
-	+	-	0	
-	-	-	0	

*Molar excess of the Tat peptides is presented as -fold excess of Tat molecules. Results are the average of duplicate wells from two experiments.

heparin at concentrations as low as 0.5 µg/ml and in a dose-dependent manner (Table 1). Cell proliferation or morphology was unchanged in the presence of heparin at concentrations as high as 5 µg/ml (data not shown). This indicated that the inhibition of viral replication is due to the binding of exogenous Tat to heparin, and not to the interference of heparin with cell growth. Heparin also inhibited the rescue effect of exogenous Tat when both heparin and Tat were added together to the cells without preincubation (Table 1). This suggested that the binding affinity of heparin and Tat is high and that heparin can block the entry of Tat protein into cells and/or nuclei and thus block its effect on provirus activation.

For bFGF, as well as other heparin-binding growth factors, and ECM proteins, binding to heparin is mediated by the basic region of these molecules [51-54]. Tat contains a similar region that mediates nuclear localization and nucleic acid binding [5]. To identify whether the binding of Tat to HSPG occurs through the basic region, competition experiments were performed with various concentrations of the Tat basic peptide amino acids 46-60, the Tat peptide amino acids 11-24 or the RGD Tat peptide amino acids 65-80. These peptides were preincubated with heparin (1 µg/ml) at 4°C for 2 h and then added to HLM-1 cells in the presence of Tat (Table 2). Heparin or Tat peptides alone or combined did not change cell proliferation, morphology, or p24 production in the absence of exogenous Tat (data not shown), but the Tat basic peptide competed out the blocking effect of heparin in a dose-dependent fashion. In contrast, the Tat peptides 11-24 or 65-80 had no effect on the block of Tat-viral rescue by heparin (Table 2). This indicated that the basic region is the Tat domain binding to heparin.

Gel shift analysis of Tat–heparin interaction and competition by bFGF

To analyze further the binding of Tat to heparin, *in vitro* binding assays were performed by gel shift analysis. Various amounts of biologically active Tat protein were mixed with [*N*-sulfonate-³⁵S]heparin sodium at 28°C for 5 min. The mixture was then treated with heparinase for 10 min and loaded into 8% non-denaturing polyacrylamide gel. As shown in Fig. 3a, Tat protein formed a complex with labeled heparin in a dose-dependent fashion, whereas no shift was observed when Tat was pretreated with unlabeled heparin (data not shown). In addition, as shown in Fig. 3b (lanes 1, 2 and 4) the binding of Tat and heparin was not competed out by an excess of BSA (1 or 10 µg) suggesting that the binding of Tat and heparin is specific. In contrast, Tat–heparin binding was competed by bFGF (Fig. 3b, lanes 2 and 3), that formed a unique complex with labeled heparin (Fig. 3b, lane 5). This indicated that Tat binds heparin specifically and suggested that Tat or bFGF binding to heparin occurs at a common site.

Purification of a highly biologically active recombinant Tat protein by heparin-affinity chromatography

Tat is very sensitive to oxidation and aggregation that leads to a rapid loss of biological activity [10,14,18,44,55]. Heparin is known to protect the function of growth factors such as bFGF and it is commonly used to purify these factors. To investigate whether the binding of Tat to heparin can protect the protein from loss of biological activity, Tat protein was expressed in *E. coli* and cell lysates were homogenized, centrifuged and the resulting supernatants were incubated with heparin–Sepharose. The bound material was then eluted with NaCl as described in Materials and methods. The total extract, flow through or after elution were then separated and analyzed by gel electrophoresis and tested by the viral rescue assay with HLM-1 cells. A single band at 14 kDa was detected from the fraction eluted at 2 M NaCl, indicating purification of Tat to homogeneity (Fig. 3c). In addition, both eluate 1 and 2 showed a very high biological activity (see legend to Fig. 3c) as compared to the best

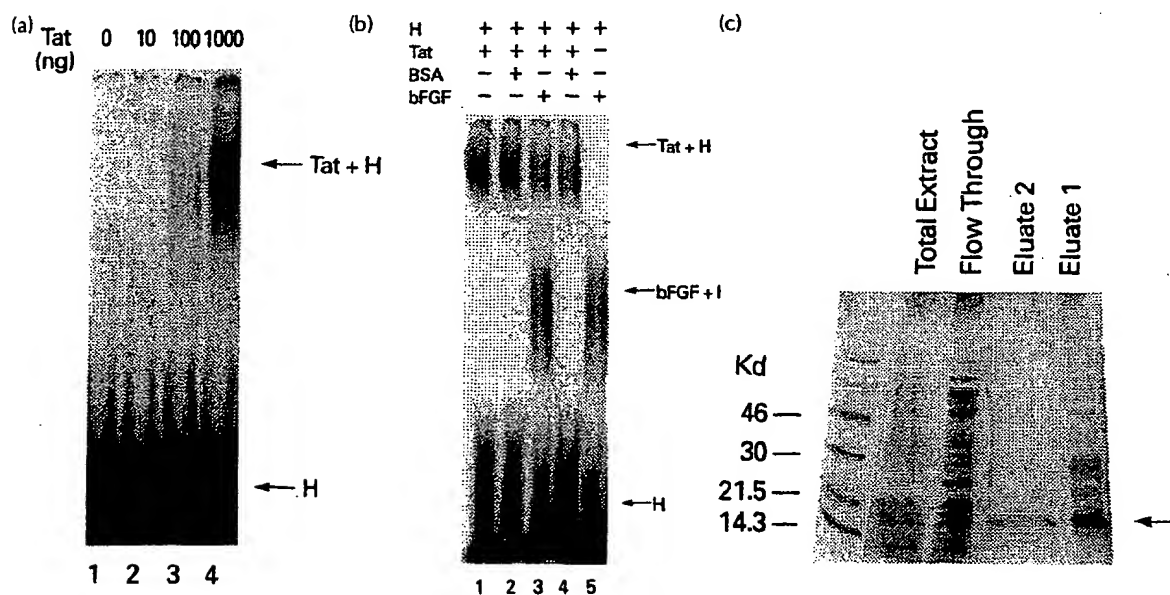


Fig. 3. (a) Tat binds heparin in a dose-dependent fashion. Biologically active Tat (0, 10, 100, or 1000 ng) was mixed with [*N*-sulfonate-³⁵S]heparin sodium in 10% fetal calf serum (FCS)–RPMI and then treated with heparinase. (b) Basic fibroblast growth factor (bFGF) but not bovine serum albumin (BSA) compete with Tat binding to heparin. Tat (75 ng) was mixed with hot heparin (lane 1). Competition experiments with bFGF (100 ng) or BSA (1 µg, lane 2, or 10 µg, lane 4) were performed in the presence or absence of Tat. The mixture was then treated with heparinase. (←), Free labeled heparin (H), complexes of Tat and heparin (Tat + H) and complexes of bFGF and heparin (bFGF + H). (+) or (–), Presence or absence, respectively, of each molecule. (c) Purification of recombinant Tat protein by heparin-affinity chromatography (representative gel from more than five independent experiments). *Escherichia coli* cell lysates were centrifuged and the supernatants incubated with heparin–Sepharose. The bound material was then eluted at 2 M NaCl (eluate 1 and 2). A fraction of total extract, flow through and eluate 1 and 2 was separated on a 10–20% polyacrylamide gel and the protein was visualized by Coomassie staining. Arrow indicates the Tat protein. Eluate 1 and 2 were tested for biological activity and compared with Tat purified by high-pressure liquid chromatography. The proteins (2 µg/ml) were tested with HLM-1 cells. Values of p24 were for eluate 1, 325 ± 2; for eluate 2, 264 ± 5; for HPLC-purified Tat, 294 ± 20.

Tat preparations obtained after expression of Tat from the same vector but purified by HPLC under reducing conditions as described previously [18,44]. This confirmed the Tat binds heparin in a fashion similar to bFGF, that is also eluted at 2 M NaCl, and indicated that heparin can protect Tat from oxidation and can allow the purification of a highly biological active protein to homogeneity in the absence of reducing agents.

Discussion

The results presented in this study indicate that Tat protein is released from *tat*-transfected cells in the absence of cell death or cell permeability changes. In contrast, Tat release requires a complete cell viability and function and a high *tat* gene expression. Release of Tat is not blocked by brefeldin A or methylamine but it is increased by a high temperature or serum-free conditions. Conversely, low temperature (18°C) reduces release of Tat. Released Tat is found in a soluble form and in a form bound to HSPG. This extracellular bound Tat can be retrieved into a soluble form by trypsin, heparin or heparinase. Tat binds heparin and this binding preserves the biological activity of Tat. At the same time, heparin blocks the biological effect of Tat. The heparin-binding property of Tat is due to its basic region that mediates the interaction with HSPG and the deposition of the protein into the ECM.

Methylamine, known to block the clustering and internalization of proteins [46], has been shown to inhibit secretion of IL-1 β , bFGF and thioredoxin [35,39,42], but not that of aFGF [38]. Similarly, the release of Tat is not inhibited by methylamine or serum-free conditions. This indicates that release of Tat does not involve recycling endosomes as previously suggested for IL-1 β [35]. However, low temperature (18°C), known to inhibit exo-endocytosis, reduces the release of Tat. In addition, stress conditions such as a high temperature and serum-free medium increase Tat release in the absence of cell death or permeability changes. This suggests that Tat may act as a heat-shock protein which is increased in response to cell damage and can protect cells from death as suggested previously [29–31]. The increase of extracellular soluble Tat observed under serum-free conditions may also be explained with the reduction of a serum component(s) that normally binds and traps the protein, rendering it unavailable for detection. The reduction of this factor(s) would increase the soluble (detectable) fraction of Tat.

The use of an alternative pathway of secretion has been suggested which would avoid the oxidizing milieu of the ER lumen [35]. Since cysteine residues present in Tat, IL-1 β and bFGF are essential for their bioactivity, the requirement of a reducing milieu for secretion is one of the reasons for alternative secretion pathways.

Recently, a synaptic vesicle protein, synaptotagmin, has been demonstrated to be associated with the release of aFGF in response to heat shock [56]. However, released aFGF is inactive, unable to bind heparin and requires activation by reducing agents. In contrast, native extracellular Tat is biologically active, as found for IL-1 β and bFGF, and can bind heparin.

Extracellular Tat is found in a soluble fraction and in a HSPG-bound fraction that can be retrieved by trypsin, heparin or heparinase, in a fashion similar to bFGF [40,41]. In particular, the presence of heparin increases the amount of soluble Tat, indicating that heparin retrieves HSPG-bound Tat and maintains it into a soluble form. It is possible that extracellular Tat and exogenous heparin form a large complex that cannot be taken up by the cells. In fact, the viral rescue effect of Tat is blocked by heparin (Table 1), whereas no inhibition of Tat transactivation is seen when Tat is scraped into cells (that allows direct entry into the cytoplasm) in the presence of heparin [47]. Since we have used the direct addition of Tat to the cells, this suggests that heparin traps the protein and interferes with its binding and uptake into cells. In addition, heparin can block 80% of the cell surface binding of Tat [47], can block cell adhesion [8] and the KS cell growth effect of Tat (G. Barillari, personal communication, 1996) that require integrin binding.

The Tat basic peptide inhibits the effect of heparin on Tat activity (Table 2) suggesting that this region mediates binding of Tat to heparin as observed for heparin-binding growth factors or ECM molecules [52–54]. In fact, bFGF can compete with Tat for heparin-binding (Fig. 3). In contrast, Tat or its basic region can displace bFGF bound to HSPG associated with the cell surface and ECM and retrieve it into a soluble form (G. Barillari, personal communication, 1996). Thus, the heparin-binding property of Tat is mediated by the basic region. Mutations in this region also increase the cytoplasmic content of the protein and its release [18]. This may be due to both reduced nuclear retention of Tat and reduced binding of Tat to HSPG with consequent increase of the soluble portion of the protein.

The binding of Tat to HSPG may have relevance *in vivo*. Endothelial and other cells do synthesize and release heparan sulfates, which have similar structure and function to heparin [57]. It has been demonstrated that complex formation between FGF and heparin protects FGF from heat, acid inactivation and proteolytic degradation [48,50]. Similarly, heparin protects Tat biological activity and allows the purification of the protein to homogeneity in a fashion similar to bFGF. In particular, most procedures for the purification of recombinant Tat involved the use of chaotropic agents and other denaturants that lead to protein unfolding which would require that purified protein being

refolded properly for biological function [14,44,55,58,59]. Tat protein is extremely susceptible to loss of biological activity during these procedures because of its high cysteine content and ease of oxidation and requires the presence of reducing agents [14,18,55]. However, the binding of Tat to heparin allows a highly-efficient one-step purification which generates a highly biologically active Tat (Fig. 3c). This indicates that *in vivo* HSPG may exert a similar protective activity and be able to store biologically active Tat.

Tat has several features in common not only with growth factors including FGF, but also with ECM molecules such as fibronectin, vitronectin, collagens and laminin [54,60–62]. Similar to these molecules, Tat has two different binding sites on the cell membrane. A low-affinity binding site described previously [47], is shown here to be mediated by the Tat basic region binding to cell-associated heparan sulfates. The other is represented by the binding of the Tat-RGD region to the integrin receptors $\alpha_5\beta_1$ and $\alpha_v\beta_3$ that mediate cell adhesion, survival, migration and invasion, and are required for cell growth induced by Tat [9–11,18]. These phenomena are further increased by or require also the binding of the Tat basic region to HSPG [8,9,11] (G. Barillari, personal communication, 1996) as found for both FGF and ECM molecules.

Since substantial quantities of Tat have been detected in sera of HIV-1-infected individuals and in KS lesions from AIDS patients, extracellular Tat may play a key role in the pathogenesis of AIDS and associated disorders [5]. Therefore, the understanding of the mechanisms of its release and extracellular fate may help in the investigation of new strategies to inhibit its activity including the use of Tat as a target for gene therapy [63] and an anti-HIV-1 vaccine [64].

Acknowledgements

We thank A. Caputo (University of Ferrara) for helpful discussions and A. Lipa for editorial assistance.

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